Microorganisms in Root Canal–treated Teeth from a German Population

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Abstract

Posttreatment apical periodontitis is usually associated with persistent or secondary intraradicular infection. This study evaluated the presence and relative levels of 28 bacterial taxa in treated root canals of teeth with posttreatment apical periodontitis from German patients using 16S ribosomal RNA (rRNA) gene probes in a reverse-capture checkerboard hybridization assay. Species-specific polymerase chain reaction (PCR) was also performed to detect *Enterococcus faecalis* and *Candida albicans*. Bacterial DNA was detected in all samples. Twenty of the 28 taxon-specific probes tested were reactive with at least one sample. Taxa detected more frequently included *Streptococcus* species (47%), *Lactobacillus* species (35%), *Dialister invisus* (29%), *Eubacterium infirmum* (29%), *Prevotella intermedia* (29%), *Selenomonas sputigena* (29%), *Synergistes* oral clone BA121 (29%), and *Treponema denticola* (29%). Only eight taxa were present at levels $>10^5$. Of these, streptococci and *T. denticola* were the most prevalent. Species-specific PCR detected *E. faecalis* in 47% of the cases and *C. albicans* in 6%. Findings of this study confirm the strong association between persistent/secondary intraradicular infection and posttreatment apical periodontitis. Most cases harbored a mixed infection, and *E. faecalis*, if present, was never the most dominant species in the consortium. Several other bacterial taxa were detected, and an involvement with the etiology of posttreatment apical periodontitis is suspected. *(J Endod 2008; 34:926–931)*

Key Words

16S rRNA gene, endodontic infection, endodontic treatment failure, polymerase chain reaction, reverse-capture checkerboard DNA-DNA hybridization

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The endodontic treatment failure is usually characterized by the presence of signs and/or symptoms of apical periodontitis, which may have persisted or emerged after treatment. A large body of evidence reveals that posttreatment apical periodontitis, like the primary disease, has an infectious etiology (1–6). However, the composition of the infecting microbiota in root canal–treated teeth is significantly different from primary infections (untreated teeth) (1, 7, 8).

Traditionally, studies on the microbiota in root canal–treated teeth evincing posttreatment apical periodontitis have been conducted using culture, which is a broad-range approach (1, 2, 9–11). These were followed by studies using molecular biology methods such as species- or group-specific polymerase chain reaction (PCR) assays focusing on cultivable bacteria (5, 6, 12, 13). A significant advance in microbiological research has been a molecular approach involving the amplification of 16S rRNA genes with "universal" bacterial primers followed by cloning to purify the mixed amplicons and sequencing to identify the taxa present (14, 15). By this broad-range molecular approach, one can build a 16S rRNA gene clone library consisting of a comprehensive inventory of sequences from both cultivable and as-yet-uncultivated bacteria present in the consortium (16). Nevertheless, technical demands and high cost can make it difficult to analyze a large number of samples by this method. Cataloging bacterial species in the oral cavity by clone libraries provides 16S rRNA gene sequence data that can be used to design primers or oligonucleotide probes to target both cultivable and as-yet-uncultivated bacteria in a large number of samples. Primers can be used in PCR assays (12), which are, however, restricted by the need to perform several individual reactions to survey several samples for the presence of several species/phylotypes. Probes can be used in molecular biology techniques suitable for large-scale clinical studies, including checkerboard hybridization assays (17).

The present study intended to evaluate the presence and relative levels of 28 bacterial taxa in treated root canals of teeth with posttreatment apical periodontitis from German patients by using a reverse-capture checkerboard hybridization assay. Target taxa for investigation included cultivable species previously linked to endodontic infections as well as newly characterized species and as-yet-uncultivated phylotypes that have been recently detected in clone libraries from periodontal or endodontic infections (18–20). Some of them have never been previously found (or even evaluated) in samples from endodontic treatment failures. Species-specific PCR was also performed to detect the prevalence of *Enterococcus faecalis* and *Candida albicans* in the same samples.

Material and Methods

Case Description

Patients attending the Department of Operative Dentistry at the Dental School of the University of Göttingen, Germany, for endodontic retreatment were selected for this study. Seventeen root canal–treated teeth (two maxillary central incisors, one maxillary lateral incisor, four maxillary premolars, two mandibular premolars, three maxillary molars, and five mandibular molars) from 13 adult white patients (seven women and six men, aged 22 to 60 years, mean age 43.5 years) were included in this study. All teeth showed radiographic evidence of apical periodontitis and had endodontic therapy completed more than 1 year earlier. All teeth were coronally restored, and no direct exposure of the root canal filling material to the oral cavity was evident. Teeth showing frank exposure of the root-filling material to the oral cavity were excluded from the

study as well as teeth in which retreatment attempts already had been initiated. Terminus of the root canal fillings ranged from 0 to 4 mm short of the radiographic apex. Twelve teeth were asymptomatic, whereas five teeth were tender to percussion. On the basis of clinical and radiographic examinations, the outcome of root canal treatment of the examined teeth was considered as unsuccessful and retreatment was indicated. The study protocol was approved by the Institutional Review Board and informed consent was obtained from all participants.

Sample Taking and DNA Isolation

After plaque removal and rubber dam application, the operative field was cleansed with 3% hydrogen peroxide and disinfected with 3% sodium hypochlorite (NaOCl), and coronal restorations were removed. Endodontic access was completed with a sterile high-speed carbide bur until the root canal filling was exposed. Whenever a post was present, it was removed by ultrasonic vibration. After completion of the endodontic access, the tooth, clamp, and adjacent rubber dam were once again disinfected with 3% NaOCl. Coronal gutta-percha was removed by means of sterile Gates-Glidden burs, and the apical root filling material was retrieved using K-type and/or Hedström files. Root canal–filling removal was always performed without the use of chemical solvents. Whenever possible, the retrieved material was transferred to cryotubes containing TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 7.6). Radiographs were taken to ensure that all filling material had been removed. A small amount of sterile saline solution was introduced into the root canal by a syringe, and the canal walls were then filed so that material could be obtained. Samples were collected by two sequential paper points placed to a level approximately 1 mm short of the root apex, based on diagnostic radiographs. No NaOCl was used as an irrigant until sampling was completed. Paper points were transferred aseptically to cryotubes containing TE buffer and immediately frozen. Samples were kept stored at −20°C and then sent all at once to Rio de Janeiro for molecular analysis.

Samples were thawed to 37°C for 10 minutes and vortexed for 30 seconds. Afterward, the microbial suspension was pelleted by centrifugation for 10 minutes at 5,000*g*. The pellet was then resuspended in 180 μL of buffer ATL supplied by QIAamp DNA Mini Kit (Qiagen, Valencia, CA), and 20 μL of proteinase K (20 mg/mL) was added. Samples were incubated for 3 hours at 56°C. Subsequently, total bacterial genomic DNA was isolated according to the protocol of QIAamp DNA Mini Kit. The total bacterial DNA was eluted with 200 μL of AE buffer (Qiagen) and stored at −20°C. DNA from a panel of several oral bacterial species was also prepared to serve as controls (12).

Broad-range PCR for Checkerboard Analysis

The reverse-capture checkerboard assay used in this study was modified from the method described by Paster et al. (17) to include some adjustments to endodontic samples. Initially, whole-genomic DNA extracts from clinical samples were used as templates in a 16S rRNA gene-based PCR protocol, which consisted of two steps. First, a practically full-length 16S rRNA gene fragment was amplified by using universal primers 8f and 1492r. Aliquots of 5 μL of the DNA extracts were used as a target in the first PCR reaction. Next, the resulting PCR product from each sample was used as a template to run two sets of partial 16S rRNA gene amplification, one using primers 8f and 519r and the other using primers 515f and 1492r. Therefore, two different fragments, which together encompass the nearly full-length 16S rRNA gene, were obtained for each sample. In these two PCR sets, the forward primers were labeled at the 5′ end with digoxigenin. This two-step heminested approach was used to achieve a better performance of PCR, particularly for samples with a low number of bacteria. Aliquots of 1 μL of the first PCR product were used in each set of the second round of amplification.

All PCR amplifications were performed in a 50 μL of reaction mixture containing 1 μmol/L concentration of each primer, 5 μL of 10× PCR buffer, 3 mmol/L $MgCl_2$, 2 U of *Tth* DNA polymerase, and 0.2 mmol/L of each deoxyribonucleoside triphosphate (all reagents from Biotools, Madrid, Spain). Negative controls consisting of sterile ultrapure water instead of sample were included with each batch of samples analyzed.

Preparations were amplified in a DNA thermocycler (Mastercycler personal; Eppendorff, Hamburg, Germany). The PCR temperature profile for the first reaction using primers 8f/1492r included an initial denaturation step at 95°C/1 min, 26 cycles at 94°C/45 seconds, 50°C/45 seconds, and 72°C/1.5 minutes and a final step at 72°C/15 min. PCR cycling conditions for the second round of amplification using primers digoxigenin-8f/519r or digoxigenin-515f/1492r included an initial denaturation step at 95°C/5 minutes, 28 cycles at 94°C/30 seconds, 55°C/1 min, 72°C/1.5 min, and final extension at 72°C/20 minutes. PCR amplicons were separated by electrophoresis in a 1.5% agarose gel, stained with 0.5 μg/mL ethidium bromide, and viewed under ultraviolet transillumination. A 100-bp DNA ladder digest (Biotools) served as the molecular size standard.

Reverse-capture Checkerboard Assay

Labeled PCR products obtained with primers 8f/519r and 515f/1492r were mixed by using equal proportions of each (45 μL) and used in a reverse-capture checkerboard assay to determine the presence and levels of 28 bacterial taxa. Probes were designed based on 16S rRNA gene sequences of the target bacteria retrieved from the GenBank, and the BLAST-based algorithm was used to verify their uniqueness against other sequences present in the GeneBank database and in a private database composed exclusively of oral 16S rRNA gene sequences. Probes were devised to have a melting temperature (T_m) of approximately 51° to 52°C and were synthesized with multiple thymidines at the 5′ end (Operon Technologies, Alameda, CA). Testing against purified DNA from the panel of oral species revealed no cross-reactions with nontargeted taxa. Two universal and three group-specific probes (*Streptococcus*, *Lactobacillus*, and *Veillonella*) were validated and reported previously (17, 21). Probe sequences are shown in Table 1.

In addition to the 28 taxon-specific probes, two universal probes were included in the assay to serve as controls. Three lanes in the membrane contained standards at the concentration of 10^5, 10^6, and 10^8 cells, which were treated the same way as the clinical samples. The sensitivity of the assay was approximately 10^3 cells.

The reverse-capture checkerboard assay was performed by using the Minislot-30 and Miniblotter-45 system (Immunetics, Cambridge, MA). First, 100 pmol of probe in TE buffer (10 mmol/L Tris HCl, 1 mmol/L EDTA, pH 8.0) were introduced into the horizontal wells of the Minislot apparatus and crosslinked to the Hybond- N+ nylon membrane (AmershamPharmacia Biotech, Buckinghamshire, England) by ultraviolet irradiation by using a Stratalinker 1800 (Stratagene, La Jolla, CA) on autocrosslink position. The polythymidine tails are preferentially crosslinked to the nylon, which leaves the specific probe available for hybridization. The membrane was then prehybridized at 55°C for 1 hour. Subsequently, 90 μL of the labeled PCR products with 50 μL of 55°C preheated hybridization solution was denatured at 95°C for 5 minutes and loaded on the membrane using the Miniblotter apparatus. Hybridization was performed at 55°C for 2 hours.

After hybridization, the membrane was washed and blocked in a buffer with casein. The membrane was sequentially incubated in anti-digoxigenin antibody conjugated with alkaline phosphatase (Roche Molecular Biochemicals, Mannheim, Germany) and ultrasensitive chemiluminescent substrate CDP Star (Roche Molecular Biochemicals).